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13. ABSTRACT (Maximum 200 Words)
The hypothesis being tested in this project is that a greater proportion of African-Americans with breast cancer harbor specific germline genetic alteration in the ATM gene or possess a particular ATM haplotype, compared to African-American women without breast cancer. An additional objective is to determine the functional impact upon the protein encoded by the ATM gene for each mutation identified.

Specific Aims: The specific aims of this project are to (1) screen 100 African-American breast cancer patients and 100 African-American women without breast cancer and (2) perform functional studies using cells from patients identified as ATM carriers to determine whether each ATM genetic variant identified affects radiosensitivity and levels of the protein encoded by the ATM gene for each mutation examined.

The main accomplishment during this past year was to accrue into the study and complete the DHPLC sequencing of 188 subjects. As a result, specific ATM variants were identified in 117 subjects. In addition, functional assays were accomplished to measure ATM kinase activity in a series of wild type cells as well as cells obtained from ATM patients, obligate ATM heterozygotes and cell lines generated from subjects accrued into this study.

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Introduction

African-American women on average present with more advanced breast cancer when compared with Caucasian women. This leads to suboptimal cure rates within this population. Numerous investigators have attempted to explain this discrepancy and determine if it stems from an inherent aggressive biologic behavior or a lack of access to appropriate medical care. It is still controversial, but there is evidence from randomized trials that when controlled for stage, African-American women have similar outcomes compared with their ethnic counterparts. However, it appears that socioeconomic factors lead to delayed screening evaluations and disease that is locally or systemically advanced rather than pre-emptive identification of early stage breast cancer. It would therefore be useful to discover a genetic marker that can serve to identify African-American women who are at increased risk for breast cancer at an age prior to disease development. The *ATM* gene has been chosen for consideration as a potential marker given its critical role in the maintenance of genomic integrity.

The hypothesis to be tested in this project is that a greater proportion of African-Americans with breast cancer harbor a genetic alteration in the *ATM* gene compared to

African-American women without breast cancer. An additional objective is to determine the functional impact upon the protein encoded by the *ATM* gene for each mutation identified.

The specific aims of this project are to (1) screen 100 African-American breast cancer patients and 100 African-American women without breast cancer and (2) perform functional studies using cells from patients identified as *ATM* carriers to determine whether each *ATM* variant identified affects radiosensitivity and levels of the protein encoded by the *ATM* gene for each mutation identified.

To accomplish this work, blood lymphocytes will be isolated from African-American breast cancer patients as well as non-breast cancer controls. DNA is isolated from these cells and each of the coding exons of the *ATM* gene for every patient will be screened for *ATM* alterations using denaturing high performance liquid chromatography (DHPLC). In those exons that display aberrant DHPLC profiles suggestive of a mutation, DNA sequencing will be performed to identify and characterize the mutation. For each person diagnosed as an *ATM* carrier, a lymphoblastoid cell line will be created to analyze ATM protein levels.

Body

Due to the substantial delay until April 30, 2003 from the HSRRB (Human Subjects Review Board) of the DOD for approval of the human subjects protocol and consent forms for this project, which was followed by an additional delay to obtain approval from both the Mount Sinai and NYU IRBs, subject accrual into this study could not be initiated until the beginning of the second year of this project. However, once these approvals were obtained, all of the study investigators made a concerted effort to rapidly accrue patients and screen DNA samples for *ATM* variants. Hence substantial progress was made in the past year of this project with complete screening of the *ATM* gene accomplished for 188 African American women, 64 of whom have been diagnosed with breast cancer and 124 age matched control subjects who have not been diagnosed with breast cancer. A full listing of the *ATM* variants detected in these patients is provided below. A full analysis of these data has not been performed to determine whether any particular variant, or group of variants, is associated with the women diagnosed with breast cancer, as this will be performed upon accrual and complete analysis of the full complement of cases and controls. However, of the total number of breast cancer subjects, 58% (27/64) were found to harbor an *ATM* variant, whereas 65% (80/124) of the controls possessed an ATM alteration. In addition, several *ATM* polymorphisms were identified that appear to be specific for African American women.

The following tables report the *ATM* variants detected for the subjects in this study;

African American Breast Cancer Patients with an *ATM* Variant

1176C>G
1254A-G
1254A-G
1254A-G
1541G-A, 4939C-T
2442C-A
2685A-G
2685A-G
2685A-G
2685A-G, 378T-A
334G-A
3383A-G
3383A-G
3383A-G
378T>A, 5557G>A
378T-A
378T-A
378T-A
378T-A
378T-A, 1176C-G
378T-A, 1176C-G, 4138C-T
378T-A, 2289T-A
378T-A, 2289T-A
378T-A, 3383A-G
4138C>T, 4400A>G
4138C-T
4279G-A
4578C-T
5557G-A
5557G-A
5557G-A
5557G-A
5557G-A, 5558A-T
5557G-A, 5558A-T
5793T>C
IVS5-7C-T, 378T-A
IVS5-7C-T, 378T-A, 4578C-T

27 African American Breast Cancer Patients did not have an *ATM* Variant.

African American Women without Breast Cancer and an *ATM* Variant

1073A-G, 2362A-C
1254A-G
1254A-G
1254A-G
1254A-G
1254A-G
1254A-G
1254A-G
1254A-G
1254A-G
1541G-A
1541G-A
1541G-A
1541G-A, 1595G-A
1541G-A, 4138C-T
1744T-C, 2610C-T
2572T-C, 2685A-G
2614C-T
2614C-T, 2685A-G
2614C-T, 2685A-G
2614C-T, 2685A-G
2614C-T, 2685A-G, 5557G-A, 6995T-C
2685A-G
2685A-G
2685A-G
2685A-G
2685A-G, 1254A-G
2685A-G, 1254A-G
2685A-G,895L-L; 1254A-G,418Q-Q
3161C-G,1054P-P
320G-A,107C-Y
334G-A, 3383A-G, 7313C-T
3383A-G, 378T-A
3383A-G
370A-G, 378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A

378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A
378T-A, 1810C-T, 6176C-T
378T-A, 2096A-G
378T-A, 544G-C
378T-A, 5557G-A
378T-A, 5557G-A
378T-A, 5793T-C
378T-A, 6176C-T
378T-A, 6235G-A, 9215A-G,
378T-A, 6235G-A, 9215A-G,
378T-A, 6437G-C, 9215A-G,
378T-A, IVS28+5G-T
378T-A; 1254A-G, 2289T-A
4138C-T
4138C-T
4258C-T
4578C-T
4578C-T
4578C-T
5557G-A
5557G-A
5557G-A
5558A-T
6235G-A
6235G-A; 9215A-G
975A-C, IVS62+8A-C
IVS5-7C-T, 378T-A
IVS5-7C-T, 378T-A
IVS5-7C-T, 378T-A

44 African American Women without Breast Cancer had no *ATM* Variant.

Additional work has been accomplished performing functional assays. Since we have only recently begun to EBV transform lymphocytes to lymphoblastoid cell lines from subjects shown to be positive for possession of an *ATM* alteration, most of the work done during this past year involved the use of cell lines derived from either cells not exhibiting an *ATM* variant (wild type), as well as cell lines derived from ataxia telangiectasia (AT) patients who possess two mutated copies of the *ATM* gene or from the parents of these AT patients who are obligate heterozygotes for a single mutated copy of the *ATM*.

The work accomplished has been to examine the functional status of the ATM protein in wild type and *ATM* mutant lymphoblastoid cell lines involving immunodetection of p53 which has been phosphorylated. Since it is known that ATM phosphorylates p53 at ser-15, and given that there are commercially available antibodies that specifically recognize p53 protein that has been phosphorylated at ser-15, it is possible to determine the kinase activity of ATM in various cell types indirectly by determining the amount of p53 in a cell that has been phosphorylated by ATM at ser-15.

Briefly, we sediment the cells and then resuspend the cells in lysis buffer (50mM Tris/HCl, 5mM Na_2EDTA, 150 mM NaCl, 0.5% NP40, 1mM DTT, 1mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM sodium orthovanadate, 1mM sodium fluoride) for 20 minutes on ice. The protein concentration is determined using a modified Bradford assay kit (RC-DC, BioRad). 1mg of cellular protein lysate is incubated with 1 µg of monoclonal anti-p53 (5 µl of a 200 µg/ml stock of 1C12 Mouse Monoclonal #2524, Cell Signaling technology) for 1 hour at 4°C with agitation. Protein-A conjugated to Sepharose beads (Sigma) is added to the mixture and incubated for 1 hr. at 4°C with agitation. The immunocomplex is precipitated by centrifugation, washed with 5 volumes of lysis buffer five times, and boiled in 20 µl Laemmli SDS-PAGE loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) (BioRad). The beads are pelleted again using centrifugation.

The supernatant (containing the protein) is loaded onto a 7.5% precast gel (BioRad) and electrophoresed until the bromophenol blue dye front reaches the end of the gel. The proteins on the gel are transferred to a PVDF membrane (Immuno-Blot, BioRad) in Towbin buffer (25mM Tris/HCl, 192 mM glycine, 20% methanol, BioRad) for 2 hr at 4°C. The membrane is allowed to dry and washed in 25 ml phosphate buffered saline with 0.1% Tween-20 (PBST) twice for 5 min at room temperature (RT). The membrane is blocked with 25 ml PBST buffer with added protein blocker for 1 hr at RT and washed twice with 25 ml PBST for 5 min at RT. To the blocked membrane is added 10 ml of antibody dilution buffer (ADB) containing polyclonal rabbit Phospho-p53 (Ser15) antibody (Cell Signaling technology #9284, diluted 1:2000 [5 µl to 10 ml ADB]) and incubated for 1 hr at 4°C with rocking. The blot is washed twice in 25 ml PBST at RT and incubated for 1 hr at 4°C with rocking in secondary antibody (goat-anti-rabbit conjugated with horseradish peroxidase [GAR-HRP]) diluted to 1:5000 in ADB (2 µl antibody added to 10 ml ADB).

The blot is washed twice in 25 ml PBST at RT. The membrane is incubated in diluted BioRad Amplification Reagent (BAR) for 10 min at RT with rocking, washed four times in PBST with 20% DMSO 5 min at RT, and twice in PBST 5 min at RT. The membrane is incubated in diluted streptavidin-HRP for 30 min, followed by two washes in PBST 5 min RT. Using the Opti-4CN colorimetric detection from the BioRad kit, 15 ml of the colorimetric solution is prepared and the blot incubated in this solution for about 30 min or until the desired level of sensitivity is attained. The blot is washed in ddH_2O for 15 min. The colorimetrically labeled blot is scanned using a table scanner and each band in the scan quantitated using ImageJ (public domain software downloaded from http://rsb.info.nih.gov/ij/).

A total of 12 separate experiments were performed with wild type cells, while cells obtained from 6 individuals diagnosed with AT in addition to 9 *ATM* heterozygotes, and the level of phosphorylated p53 measured using densitometry. Cells were irradiated with either 0 or 4 Gy of x-rays and incubated either 0.5 or 2 hr. The densitometric results for each time point were divided by the value in each experiment for unirradiated cells to normalize these results. The mean values (with standard deviations) for wild type cells incubated either 0.5 or 2.0 hr were 3.9±1.9 and 8.3±4.4. In contrast, the mean values for cells obtained from AT patients at 0.5 and 2.0 hr, were 1.2±0.5 and 3.8±2.2. Hence, as expected, the AT cells displayed significantly less p53 phosphorylation compared with wild type cells following a 0.5 hr incubation. Although the value obtained after a 2 hr incubation was lower for the AT compared with wild type cells, there was not a statistically significant difference. In contrast, the mean ratios at 0.5 and 2 hr for obligate heterozygotes were 4.1±1.9 and 7.1±4.9. Hence, similar values were found for heterozygotes compared with wild type cells. These experiments provide a foundation for analysis of the *ATM* variant cell lines that are being generated in this project

In addition, p53 phosphorylation was measured in three cell lines obtained using lymphoblastoid cell lines created from the African American subjects in this study, the results are as follows for cases (women diagnosed with breast cancer) and controls (women not been diagnosed with breast cancer);

Patient Description	Variant	p53-phosphorylated	
		0.5 hr	2.0 hr
Case-Breast cancer	IVS5-7C>T	4.8	9.0
Control-No breast cancer	2096A>G	2.9	6.2
Control-No breast cancer	6176C>T	2.5	4.7

Hence, for two of these three cell lines, the ability of ATM to phosphorylate p53 appears somewhat deficient compared with wild type cells. These experiments will be repeated two additional times to determine whether there are statistically significant differences between any of these cell lines from either wild type or AT cells

Key Research Accomplishments

- Accrual and complete DHPLC screening of the *ATM* gene in 188 African American subjects, 64 of whom have been diagnosed with breast cancer and 124 age-matched controls who have not been diagnosed with breast cancer.
- Identification, through DNA sequencing, of the specific *ATM* genetic variants in 117 subjects.
- Of the African American women diagnosed with breast cancer, 58% (27/64) were found to harbor an *ATM* variant, whereas 65% (80/124) of the controls possessed an *ATM* alteration.
- Identification of novel polymorphisms in the *ATM* gene of African American women.
- Measurement of ATM functional activity through performance of western blots to measure p53 ser-15 phosphorylation with a series of wild type, AT and ATM heterozygote cell lines in addition to three cell lines generated from subjects accrued into this study.

Reportable Outcomes

None

Conclusions

A complete analysis of the results to determine whether there are specific genetic alterations or haplotypes associated with breast cancer patients, will not be performed until the full complement of cases and controls are accrued and screened. However, it has been found in these initial results that 58% (27/64) of the African American women diagnosed with breast cancer were found to harbor an *ATM* variant, whereas 65% (80/124) of the controls possessed an *ATM* alteration. Interestingly, it appears from these preliminary results that several *ATM* variants may be specifically associated with subjects not having breast cancer and therefore may confer a protective effect. In addition, several novel

During the remainder of this project, genetic screening of the full complement of case and control subjects will be accomplished in addition to performance of the functional assays with lymphoblastoid cell lines generated from lymphocytes derived from subjects in this study to establish which of the *ATM* variants identified affect the activity of the ATM protein.

References

None

Appendices

None